Research Note

Isolation of DNA for PCR Assays from Noncultivable *Campylobacter jejuni* Isolates

K. A. Callicott,[1] N. J. Stern, K. L. Hiett, and the Campy-on-Ice Consortium[2]

United States Department of Agriculture, Agricultural Research Service, Poultry Microbiological Safety Research Unit, Athens, Georgia 30605

ABSTRACT Isolates of *Campylobacter jejuni* shipped internationally often arrive in a noncultivable state. We describe a PCR-based methodology whereby phylogenetic information can be recovered from noncultivable *C. jejuni* stored in Wang's transport medium. The robustness of this methodology was initially tested using 5 previously characterized strains of *C. jejuni* isolated from various sources associated with poultry production. These isolates were stored in Wang's transport medium before being subjected to 1 of 5 treatments designed to render the stored cells noncultivable: prolonged storage at room temperature, prolonged incubation at 42°C, multiple rounds of freezing and thawing, boiling, or contamination with *Pseudomonas aeruginosa* (ATCC 27853). This method resulted in DNA appropriate for PCR. An approximately 400-nucleotide amplicon from the *fla*A gene and an approximately 800-nucleotide amplicon from 16S rDNA were readily obtained, and a 1.5-kb section of the *fla*A locus was amplified from about half of the samples. These results indicate that this method may be useful for isolate typing schemes based on PCR amplification of *Campylobacter* DNA, including flaA short variable region (*fla*A SVR) sequencing, multilocus sequence typing (MLST), and *fla*A PCR-RFLP. By using this method, isolates unrecoverable from transport medium can still be used to provide phylogenetic information for epidemiological studies.

(*Key words*: *Campylobacter,* DNA preparation, genotyping)

2005 Poultry Science 84:1530–1532

INTRODUCTION

Campylobacter is the most frequent cause of bacterial food poisoning in developed countries, and poultry is one of the main sources for *Campylobacter* infection in human illness (Friedman et al., 2000). As such, the medical and poultry production communities have an interest in the epidemiology of this genus of bacteria. Yet, to study globally distributed organisms such as *Campylobacter* spp., isolates must often be shipped internationally. Unfortunately, even isolates stored in transport medium (Wang et al., 1980) sometimes arrive at their destination in a noncultivable state due to the stresses of travel or to prolonged delays in delivery. We describe a simple method of DNA preparation from noncultivable isolates stored in Wang's transport medium.

MATERIALS AND METHODS

Inoculation and Growth Conditions

Enriched *Brucella*[3] transport medium (Wang's transport medium) was prepared according to the recipe in Wang et al. (1980), with the exception that lysed horse blood[4] was used in place of defibrinated sheep blood. Five *Campylobacter jejuni* isolates (AL02, AL50, AL56, AL62, and ALP46) were chosen from among isolates described in Hiett et al. (2002). For each isolate, 10 tubes of Wang's transport medium were inoculated using a 10-μL loop from a 48 h at 42°C lawn (in excess of 10^8 cells) and incubated for 24 h at 42°C. The presence of cultivable

Received for publication November 30, 2004.

Accepted for publication June 30, 2005.

[1]To whom correspondence should be addressed: kcallicott@saa.ars.usda.gov.

[2]The Campy-on-Ice Consortium consists of H. Briem (Directorate of Health, Reykjavik, Iceland), V. Fridricksdóttir (Institute of Experimental Pathology, Reykjavik, Iceland), F. Georgsson (Food Laboratory, Environmental and Food Agency of Iceland, Reykjavik, Iceland), E. Gunnarsson (Institute of Experimental Pathology, Reykjavik, Iceland), H. Harðardóttir (National University Hospital of Iceland, Reykjavik, Iceland), K. Kristinsson (National University Hospital of Iceland), J. Reiersen (Chief Veterinary Office, Reykjavik, Iceland), E. Berndtson (SweChick, Kristianstad, Sweden), J.-R. Bisaillon (Canadian Food Inspection Agency, Ottawa, Ontario, Canada), V. Edge (Health Canada Center for Infectious Disease Prevention and Control, Guelph, Ontario, Canada), A. Fazil (Health Canada Laboratory for Foodborne Zoonoses, Guelph, Ontario, Canada), P. Michel (Health Canada Laboratory for Foodborne Zoonoses), G. Paoli (Decisionalysis Risk Consultants, Inc., Ottawa, Ontario, Canada), R. Lowman (Canadian Food Inspection Agency, Ottawa, Ontario, Canada), K Callicott (USDA-Agricultural Research Service), K. Hiett (USDA-Agricultural Research Service), and N. Stern (USDA-Agricultural Research Service).

[3]Catalog number 7121, Acumedia Manufacturers, Inc., Baltimore MD.

[4]Catalog number L1-010, Lampire Biological Laboratories, Pipersville, PA.

Campylobacter was verified in each tube by streaking onto Campy-Cefex plates (Stern et al., 1992). Each tube was then subject to 1 of 5 treatments designed to render the *C. jejuni* compromised or noncultivable.

In the first treatment (group A), the inoculated tubes were held at room temperature in a Saf-T-Pak box[5] in the laboratory. Every 2 wk, the cultures were streaked onto Campy-Cefex plates to verify the presence or absence of cultivable *Campylobacter jejuni.* Approximately 4 mo were required for all tubes to test as noncultivable. After all tubes were found to be noncultivable, DNA was extracted using the protocol below.

In the second treatment (group B), the tubes were held at –20°C for approximately 48 h, thawed, and then checked for cultivable *Campylobacter* by streaking onto Campy-Cefex plates. Tubes were refrozen when *Campylobacter* colonies were observed on the plates. This freeze-thaw cycle was repeated every 3 d until no *Campylobacter* colonies were observed after streaking. This required 3 freeze-thaw cycles, after which the tubes were frozen until DNA was extracted.

In the third treatment (group C), inoculated tubes were boiled for 15 min at 100°C. The lack of cultivable *Campylobacter* was verified by streaking onto Campy-Cefex plates. The tubes were then held at 4°C until DNA was extracted.

In the fourth treatment (group D), the inoculated tubes were held at a constant 42°C. Every 2 wk, the tubes were checked for cultivable *Campylobacter* by streaking onto Campy-Cefex plates. After about 4 mo, none of the 10 tubes remained cultivable. DNA was then extracted from the Wang's medium.

In the last treatment (group E), *C. jejuni* inoculated tubes were contaminated by adding a 10-μL loop from a 24 h at 37°C lawn (in excess of 10^9 cells) of *Pseudomonas aeruginosa* ATCC 27853[6] to the transport tubes. After incubation at 42°C for 1 d, a loop of Wang's transport medium from each tube was streaked onto a Campy-Cefex plate to verify the presence of *Campylobacter* and *Pseudomonas* colonies. These tubes were then held at 4°C until DNA was extracted from all 50 tubes in this experiment.

DNA Isolation

With a sterile micropipette tip, 40 μL of inoculated Wang's transport medium was drawn up, taking care to avoid air bubbles and agar clumps. This aliquot was added to 200 μL of sterile, distilled water in a 0.5-mL microfuge tube. The microfuge tube was tightly capped and boiled at 100°C for 5 min. The tube was cooled on ice, spun briefly at high speed in a microcentrifuge to precipitate cellular fragments, and stored at –20°C for later analysis.

[5]Catalog number STP-100, Saf-T-Pak, Inc., Edmonton, Alberta, Canada.

[6]American Type Culture Collection, Rockville, MD.

[7]Catalog number N808-0153, Applied Biosystems, Inc., Foster City, CA.

[8]Catalog number N808-0260, Applied Biosystems, Inc.

TABLE 1. Amplification success for different PCR targets from DNA prepared by boiling Wang's transport medium

PCR target	Approximate product size	Number positive
flaA SVR	~400 bp	33/50
16S	~800 bp	42/50
flaA	~1.5 kb	29/50

PCR Amplification

The *fla*A SVR was amplified using 0.125 μ*M* of the primers FLA242FU (5′-CTATGGATGAGCAATTWAAAAT-3′) and FLA625RU (5′-CAAGWCCTGTTCCWACTGAAG-3′; Meinersmann et al., 1997) in 50-μL reactions containing 10 μL of DNA prepared from Wang's transport medium, 1× PCR Buffer II,[7] 3.0 m*M* $MgCl_2$,[7] 0.8 m*M* each dNTP,[8] and 1.25 U of AmpliTaq DNA Polymerase.[7] These conditions have been slightly modified from those described in Meinersmann et al. (1997), as we have found that the above conditions more reliably amplify from a greater variety of *C. jejuni* isolates (data not shown). The reactions were subjected to 36 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, followed by a 5-min extension at 72°C.

Amplification of a 0.8-kb section of 16S ribosomal RNA used 0.125 μ*M* of the primers 8FPL (5′-AGTTTGATCCTGGCTCAG-3′) and 806R (5′-GGACTACCAGGGTATCTAAT-3′; Relman, 1993) in 100-μL reactions containing 10 μL of DNA, 1× PCR Buffer II, 3.0 m*M* $MgCl_2$, 0.8 m*M* of each dNTP, and 2.5 U of AmpliTaq DNA Polymerase. After the reagents were added to the reaction tube but before the DNA was added, the reaction tubes were exposed to ultraviolet irradiation for 1 min to control for any potential contamination. The reactions were subjected to 40 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, followed by a 5-min extension at 72°C.

The amplification of a 1.5-kb section of the *fla*A gene used 0.125 μ*M* of the primers FLA242FU and FLA1728R (5′-CTGTAGTAATCTTAAAACATTTTG-3′; Nachamkin et al., 1993) in 50-μL reactions containing 10 μL of DNA, 1× PCR Buffer II, 3.0 m*M* $MgCl^2$, 0.8 m*M* of each dNTP, and 1.25 U of AmpliTaq DNA Polymerase. The reactions were subjected to 94°C for 1 min followed by 35 cycles of 92°C for 30 s, 50°C for 1 min, and 72°C for 2.5 min. This was followed by a final extension at 72°C for 5 min.

RESULTS AND DISCUSSION

The PCR amplification of DNA samples prepared from transport medium was generally successful using 3 sets of primers (Table 1). The strongest result came from amplification of a portion of the 16S ribosomal DNA (rDNA), where 84% of the samples amplified. When the *fla*A SVR primers were used, 66% of the samples amplified. Although the 16S PCR produces a larger amplicon than the *fla*A SVR, there are 3 copies of the ribosomal genes on the *Campylobacter jejuni* chromosome (Parkhill et al., 2000). This may make it less sensitive to shearing of the template

DNA. Finally, 58% of the samples could be amplified with the primers for the larger segment of the *fla*A gene, although only 17 samples (34%) showed strong amplification (data not shown).

Because of the inhibitory nature of blood samples (Abu al-Soud and Rådström, 2001), reaction conditions may need to be optimized. The reamplification of a particular DNA preparation will sometimes yield a fragment in cases when some of the protein and cellular debris have been aspirated with the DNA. When that fails, a second preparation of DNA from isolates that do not amplify sometimes proves fruitful. The transport medium is not a homogeneous solution, and sometimes clumps of agar can decrease the actual amount of transport medium used for DNA preparation. Of the 16 DNA preparations that did not initially amplify using *fla*A SVR primers, 5 samples amplified when we tried the PCR a second time on the same DNA preparations. When we prepared fresh DNA from the samples that still did not work, an additional 3 amplified, bringing the total to 42 amplicons from 50 samples.

During the course of a large *Campylobacter* epidemiology study in Iceland, we received 10,211 isolates, and 4,897 isolates arrived in an unrecoverable state due to shipping mishaps. To date we have attempted to amplify *fla*A SVR from 679 samples, and we have been able to successfully amplify and sequence 674 samples from the first or second DNA preparation.

Although it would be far preferable to receive viable cells to study, delays in delivery, lost packages, and customs hold-ups can render the most carefully prepared isolates unusable. In this situation, the above protocol provides a simple means for generating genetic marker information from samples of *Campylobacter* that are no longer recoverable. Due to the lower quality of the DNA, this is not appropriate for genetic markers that depend on unsheared DNA, such as pulse field gel electrophoresis, amplified fragment length polymorphisms, or large PCR targets. However, this protocol provides a simple method for producing DNA appropriate for shorter PCR targets and preserves epidemiologically important genetic information in an inexpensive manner. Although we have not tested this method in other transport media, such as Cary-Blair (Cary and Blair, 1964), we foresee no inherent reasons why it could not also be used in those situations.

ACKNOWLEDGMENTS

The project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 0202316. We thank Latoya Wiggins (USDA-Agricultural Research Service) for her technical assistance. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

REFERENCES

Abual-Soud, W., and P. Rådström. 2001. Purification and characterization of PCR-inhibitory components in blood cells. J. Clin. Microbiol. 39:485–493.

Cary, S. G., and E. B. Blair. 1964. New transport medium for shipments of clinical specimens: I. Fecal specimens. J. Bacteriol. 88:96–98.

Friedman, C. R., J. Neimann, H. C. Wegener, and R. V. Tauxe. 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. Pages 121–138 in *Campylobacter*. 2nd ed. I. Nachamkin and M. J. Blaser, ed. ASM Press, Washington, DC.

Hiett, K. L., N. J. Stern, P. Fedorka-Cray, N. A. Cox, M. T. Musgrove, and S. Ladely. 2002. Molecular Subtype analyses of *Campylobacter* spp. from Arkansas and California poultry operations. Appl. Environ. Microbiol. 68:6220–6236.

Meinersmann, R. J., L. O. Helsel, P. I. Fields, and K. L. Hiett. 1997. Discrimination of *Campylobacter jejuni* isolates by *fla* gene sequencing. J. Clin. Microbiol. 35:2810–2814.

Nachamkin, I., K. Bohachick, and C. M. Patton. 1993. Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. J. Clin. Microbiol. 31:1531–1536.

Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quall, M.-A. Rajandream, K. M. Rutherford, A. H. M. van Vliet, S. Whitehead, and B. G. Barrell. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665–668.

Relman, D. A. 1993. Universal bacterial 16S rDNA amplification and sequencing. Pages 459–495 in Diagnostic Molecular Microbiology: Principles and Applications. D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White, ed. ASM Press, Washington, DC.

Stern, N. J., B. Wojton, and K. Kwiatek. 1992. A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni*. J. Food Prot. 55:514–517.

Wang, W.-L. L., N. W. Luechtefeld, L. B. Reller, and M. J. Blaser. 1980. Enriched Brucella medium for storage and transport of cultures of *Campylobacter fetus* subsp. *jejuni*. J. Clin. Microbiol. 12:479–480.